Genetic diversity and sex determination in date palms (*Phoenix dactylifera* L.) based on DNA markers

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Moghaieb R. E.A. *, Abdel-Hadi A.A. *, Ahmed M. R.A. ** and Hassan A. G. M. **

*Department of Genetics and Genetic Engineering Research Center (GERC), Faculty of Agriculture, Cairo University, Giza, Egypt

** Plant Tissue Culture Unit, Desert Research Center (DRC), Ministry of Agriculture, Egypt

ABSTRACT

Genetic diversity and relatedness were measured among six date palm (Phoenix dactylifera L.) genotypes by means of RAPD and inter-simple sequence repeat (ISSR) analyses. The level of polymorphism among the six genotypes as revealed by RAPD and ISSR was 60.2% and 73%, respectively. Based on the data obtained, it was possible to discriminate between the different genotypes used and to identify the unknown genotype collected from Matroh Governorate. The data indicated that ten RAPD and six ISSR markers were found to be genotype-specific. The highest number of RAPD specific markers was scored for the unknown genotype (5 markers) while the cultivar Karamah and Frehi scored three and two markers, respectively. Four ISSR markers characterize the cultivar Karamah where Frehi and Oshkingbil cultivars were characterized by one marker each. The cluster analysis indicates that the unknown cultivar was closely related to the cultivar Frehi and Oshkingbil. The potential uses of the biochemical and molecular markers in sex identification of in vivo grown date palm were investigated. Two male specific protein bands with the molecular weight of 72 and 47 kDa and another two female specific bands with the molecular weight of 135 and 55 kDa were detected. Female specific peroxidase band (RF of 0.4) and male specific estrase band (RF of 0.5) were also detected. Random amplified polymorphic DNA (RAPD) technique was used to compare genetic material from male and female date palm trees. The sex-specific RAPD markers were determined. The data indicate that 13 RAPD markers were found to be female specific and 5 markers were male specific and can be applied to screen male and female plants at young stages.

Key words: *Date palm, ISSR, RAPD, sex-specific markers.*

INTRODUCTION

Date palm is a dioecious, perennial monocot plant that is commercially important in the Middle East and North Africa. The entire tree of date palm is utilized to provide food, shelter, fiber, clothing, furniture and many other-products. Improvement of date palm is very difficult due to its long life cycle, strongly heterozygous nature and impossibility to determine sex at early stages of development (Moursy and Saker, 1996).

The analyses of genetic variation and relatedness in germplasm are of great value for genetic resources conservation and plant breeding programs to determine the best crosses between different genotypes. Over the

years, the methods for assessing genetic diversity ranged from classical strategies such as morphological analysis to biochemical and molecular techniques (Demissie *et al.*, 1998).

Knowledge of genetic diversity of the genetic resources is crucial for breeders to better understand the evolutionary and genetic relationships among accessions, to select germplasm in a more systemic and effective fashion, and to develop strategies to incorporate useful diversity in their breeding programs. These genetic diversity measures can be used to maximize the level of variation present in segregating populations by crossing genotypes with greater genetic distance (Paterson *et al.*, 1991 and Li and Nelson, 2001).

Molecular markers are currently being applied in genetic diversity studies because, unlike morphological characteristics, they are not affected by environmental variation. Molecular markers have many uses, including the development of molecular genetic maps and for variety identification (Cullis, 2002). The evolution of these markers has been started with restriction fragment length polymorphisms (RFLP) (Saiki *et al.*, 1985) detected by Southern hybridization, but this technique is laborious and involves expensive and radioactive/toxic reagents. Other molecular markers, such as random amplified polymorphic DNA (RFLP) Williams *et al.*, 1990), amplified fragment length polymorphisms (AFLP) Karp *et al.*, 1996), microsatellites (SSR) Zane *et al.*, 2002) and the ISSR technique are powerful, rapid, simple, reproducible and inexpensive ways to assess genetic diversity or to identify closely related cultivars in many species, including fruit trees (Reddy *et al.* 2002).

A wide range of marker techniques has already been applied to distinguish between varieties of the date palm, *Phoenix dactylifera* L., including RAPDs (Saker *et al.*, 2006 and Ahmed *et al.*, 2009), AFLPs (Devanand and Chao, 2003 and Rhouma *et al.*, 2007) and SSRs (Bilotte *et al.*, 2004 and Zehdi *et al.*, 2004 and b).

In date palm, a major problem for farmers is to identify the sex of saplings at an early stage so that they can cultivate in their orchards a sufficiently large number of productive female trees with only a minimal number of males. However, attempts to identify the sex of the dioecious species at an early stage have remained frustratingly unsuccessful. In recent years, there have been serious efforts to understand the genetic basis of sex determination in plants and to develop methods to identify sex at an early stage by using molecular marker tools (Mulcahy *et al.*, 1992; Hormaza *et al.*, 1994; Biffi *et al.*, 1995; Younis *et al.*, 2008 and Mohasseb *et al.*, 2009)

The objectives of the present study were to genetically identify an unknown date palm cultivar grown at Matroh Governorate compared with the other known cultivars grown in the same area based on RAPD and ISSR analyses. The other objective was to find out biochemical and molecular sex-specific markers that can be used to distinguish between male and female date palms to facilitate the selection and identification of good male pollinators for further using in breeding programs aiming to improve the quality of date fruits.

MATERIALS AND METHODS

I-Cultivar identification

Plant Material

Young leaf samples were obtained from six adult date palm tree (*Phoenix dactylifera* L.) cultivars growing in open fields in Wadi Abu-Lahw and Siwa Oasis, Matroh Governorate, Egypt. The cultivars name and the respective locations are represented in Table (1).

Table (1): Cultivar name, sampling place and fruit characteristics of six date palm genotypes collected from Matroh Government.

No.	Cultivar name	Source	Fruit characteristic
1	Unknown	Wadi Abu Lahw	Semi dry
2	Frehi	Siwa Oasis	Semi dry
3	Oshkingbil	Siwa Oasis	Semi dry
4	Azzawy	Siwa Oasis	Dry
5	Siwy	Siwa Oasis	Semi dry
6	Karamah	Siwa Oasis	Dry

Table (2): RAPD and ISSR primers name and sequence used to study the genetic diversity among six date palm genotypes.

RAPD analysis		ISSR analysis		
Primer name	Sequence	Primer name	Sequence (5' to 3')	Ann. Temp.
OP- A1	5'- CAGGCCCTTC -3'	P1	GAG(CAA)5	55 °C
OP- A4	5'- AATCGGGCTG -3'	P4	CTG(GT)8	55 °C
OP - B05	5'- TGCGCCCTTC -3'	P6	(AG)8GCC	54 °C
OP- B10	5'- CTGCTGGGAC -3'	P7	(AG)8GTG	54 °C
OP- C8	5'- TGGACCGGTG -3'	P8	(GA)8ACC	54 °C
OP- D10	5'- GGTCTACACC -3'	P9	(GA)8ATC	54 °C
OP- G14	5'- GGATGAGACC -3'			
OP- K2	5'- GTCTCCGCAA -3'			
OP- K4	5'- CCGCCCAAAC -3'			
OP- K10	5'- GTGCAACGTG -3'			
OP- P.03	5'- CTGATACGCC -3'			

DNA isolation

Total genomic DNA was isolated using the method descried in Rogers and Bendich (1985). A set of out of twenty-two random 10-mer primers (Operon Technology, Inc., Alameda, CA, USA) tested was used in the detection of polymorphism among the six date palm genotypes. The names and sequences of the primers that gave reproducible results are listed in Table (2).

RAPD analysis

The reaction mixture (20 µl) contained 10 ng DNA, 200 µM dNTPs, 1 M primer, 0.5 units of Red Hot Taq polymerase (AB-gene Housse, UK) and 10-X Taq polymerase buffer (AB-gene Housse, UK). Samples were heated to 94 °C for 5 min and then subjected to 35 cycles of 1 min at 94 °C; 1 min at 35C and 1 min at 72 °C. The amplification products were separated in 1% (w/v) agarose gel in 1 x TBE buffer and visualized by staining with ethidium bromide.

ISSR analysis

The technique was carried out according to Adawy *et al.* (2002 and 2004). Six

oligonucleotides namely P1, P4, P6, P7, P8 and P9 described by Bornet and Branchard (2004) were used as generic primers in PCR amplification of inter simple sequence repeat regions (Table 2). PCR was performed in 20 µl reaction volume containing 1X PCR buffer, 1.75 mM MgCl2, 5 mM of each dNTPs, 40 µM oligonucleotide primer, 25ng genomic DNA and 1 U of Taq DNA polymerase (AB-gene Housse, UK). A high stringency touchdown and hot start thermo cycling profile was used as follows: an initial denaturation step for 5 min at 94ºC followed by ten touch down cycles (94ºC/30 sec, 65-55ºC/45 sec, 72ºC/1 min). This was followed by thirty-five cycles (94ºC/30 sec, 55ºC/45 sec, 72ºC/1 min) and then a final extension cycle at 72ºC for 7 min. The PCR products were separated on 2% agarose gel in 1X TBE buffer containing ethidium bromide and photographed with a Polaroid camera.

Band scoring and cluster analysis

The RAPD and ISSR gel images were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v. 4.0.1 (Bio-Rad Laboratories, Hercules, CA, USA). The bands were sized and then binary coded by 1 or 0 for their presence or absence in each genotype. The systat ver. 7 (SSPSS inc.c 1997 spss inc.3/97 standard version) computer program was used to calculate the pairwise differences matrix and plot the dendrogram among date palm genotypes (Yang and Quiros, 1993). Cluster analysis was based on similarity matrices obtained with the unweighed pair-group method (UPGMA) using the arithmetic average to estimate the phenogram.

II- Sex differentiation in date palm based on biochemical and RAPD markers

Protein analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for total storage proteins extracted from young leaf samples representing both male and female trees according to the method described by Laemmli (1970).

Isozyme analysis

Isozyme extraction was performed using young leaf samples representing both of male and female trees. Tissue (400 mg) was ground in 2 ml extraction buffer (0.1% (w/v) Tris-citric acid, pH 7.5; 1% (w/v) polyvinyl pyrolidone (PVP); 0.1% (w/v) ascorbic acid and 0.1% (w/v) cysteine) and centrifuged at 5333 xg (JS - 5.2 rotor), at 4 °C for 5 min. Twenty µl of extracted samples were used for electrophoresis on polyacrylamide gel (SDS-PAGE) according to the method of Stegmann *et al.* (1983), using Pharmacia electrophoresis apparatus (GE-4).

Peroxidase was detected by incubating the gel in darkness for one hour at 37°C in a mixture of 15 ml of 10% benzidine (in 95% ethanol); 85 ml of 1mM potassium acetate and 1 ml of 1% H_2O_2 (pH 4.7). After the incubation period, the gel was rinsed in distilled water and fixed in 50% glycerol for one hour. Esterase was detected by incubating the gel in darkness for one hour at 37°C in a mixture of 100 ml (0.15 M) phosphate buffer (pH 7.2) containing 20 mg 1-naphthylacetate dissolved in 2 ml acetone and 50 mg fast blue RR salt. After the incubation period the gel was rinsed in distilled water and fixed in 50% glycerol for one hour prior to scoring and photography. Rf value of each band was calculated as follows:

$$\mathbf{Rf} = \frac{\text{Distance traveled by the band from the top of the running gel}}{\text{Distance traveled by the tracking dye}}$$

RAPD analysis

Total genomic DNA was isolated from male and female date palm trees using the method descried by Rogers and Bendich (1985). A set of eight primers namely A1, A4, B5, C8, D10, G14, K2 and K4 were used. The primers sequences are listed in Table (2).The PCR conditions and data analysis were carried out as described above.

RESULTS AND DISCUSSION

Cultivar identification

Molecular markers have proved to be useful, both in the identification of individual varieties and in the development of phylogenetic relationships among plant varieties. Molecular markers have to be developed for each species, from genomic regions that have undergone sufficiently rapid evolutionary change, in order to be useful for discriminating among closely-related germplasm (Zehdi *et al.,* 2004a). In order to investigate the genetic polymorphism among six date palm cultivars and to determine the genetic relationship between the unknown date palm cultivar collected from Matroh Govern orate with some of the known cultivars from the same area, random amplified polymorphic DNA (RAPD) and ISSR analyses were performed. Ten out of twenty two primers tested resulted in the appearance of polymorphic and reproducible PCR products. In this respect, Sedra *et al.* (1998) reported that 19 out of 123 prescreened arbitrary decamer primers revealed polymorphic and reproducible results.

This study showed that a total of 93 RAPD markers was detected among the six date palm cultivars of which, 56 bands were polymorphic (60.2%) and can be considered as useful RAPD markers for the date palm cultivars used (Fig.1 and Table 3). In contrary, Hussein *et al.* (2005) reported that the analysis of 14 date palm accession by RAPD revealed a very low intra-varietal polymorphism (25.2%).

Primer name	Total scorable bands	Polymorphic bands	Polymorphism%
OP- A1	12	4	57.1
OP- A4	9	6	66.6
OP- B10	8	3	37.5
OP- C8	10	8	44.4
OP- D10	11	7	50
OP- G14	5	1	20
OP- K2	9	6	44.4
OP- K4	9	5	50
OP- K10	10	8	55.5
OP- P.03	10	8	62.5
Total	93	56	60.2

Table(3): Genetic polymorphism among six date palm genotypes as revealed by RAPD analysis.

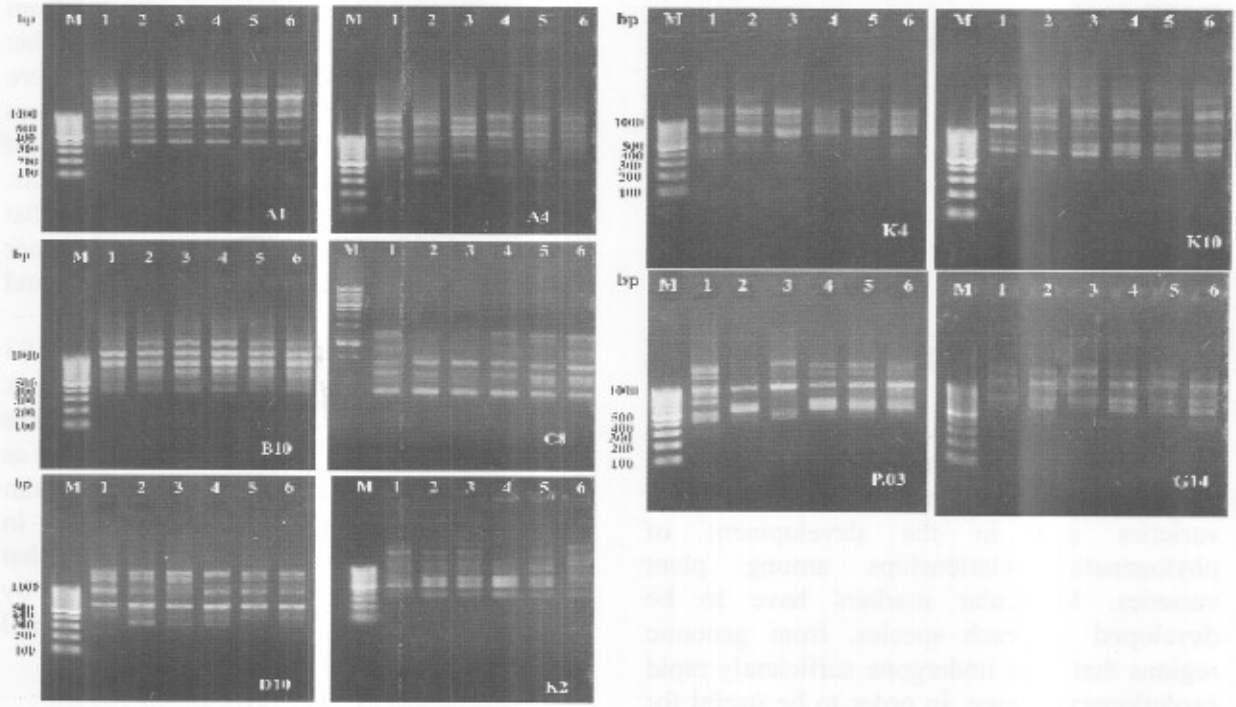

Fig. (1): RAPD banding patterns of six date palm genotypes using ten selected random primers, M: 1 kbp plus DNA ladder, 1-6: are the unknown, Frehi, Oshkingbil, Azzawy, Siwy and Karamah cultivars, respectively.

Genetic markers such as AFLP, SSR and ISSR have been applied to assess molecular polymorphism (Gupta and Varshney 2000, El-Assar *et al.*, 2005and Ahmed and Al-Qaradawi 2009). The inter-simple sequence repeat (ISSR) analysis was developed based on DNA amplification with a single 15 to 20-bp primer homologous to a microsatellite repeat and has a short (1- 4 bp) random degenerated sequence (an anchor) at the 3` or 5` end. These ISSR primers allow DNA amplification of regions located between two closely spaced, oppositely oriented SSRs, yielding a reproducible pattern of genomic fragments, which is similar to a RAPD pattern but usually includes more bands and is more reproducible. Therefore, ISSRs have a high capacity to reveal polymorphism and offer great potential to determine genetic diversity at inter and intra-specific levels as compared to other arbitrary primers (Reddy *et al.*, 2002).

In the present study six ISSR primers were used to investigate the level of polymorphism among the six date palm genotypes. The data presented in Fig.(2) show the ISSR banding pattern of the different date palm genotypes tested. All primers produced fragments, and showed different levels of polymorphism (Fig. 2 and Table 4). The data indicate that 57 out of the 78 band generated were polymorphic (73 %) (Table 4).

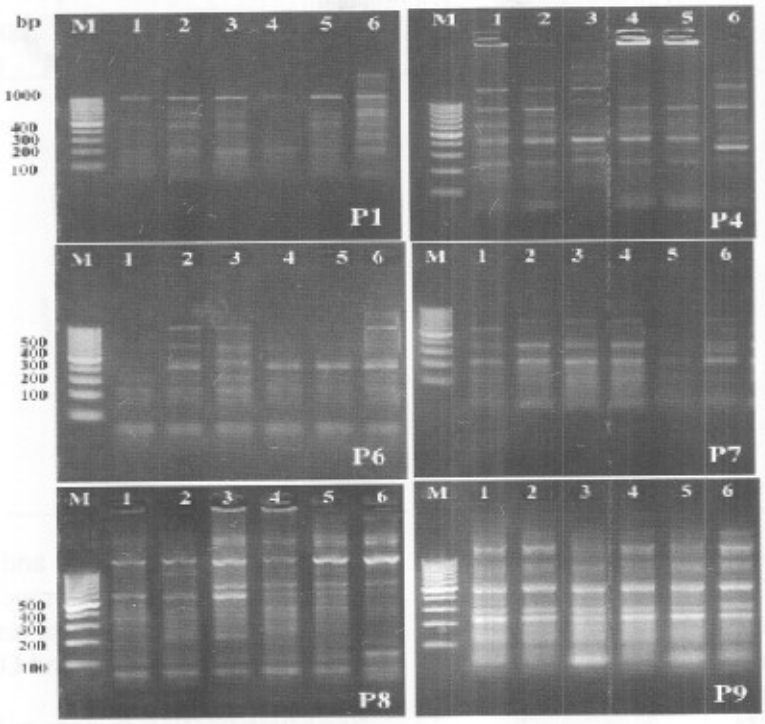

Fig. (2): ISSR banding patterns of the six date palm cultivars used. M: 1 kbp plus DNA ladder, 1-6: are the unknown, Frehi, Oshkingbil, Azzawy, Siwy and Karamah cultivars, respectively.

Table (4): ISSR primer names, total and polymorphic bands generated among the six date palm genotypes used.

Primer name	Total scorable bands	Polymorphic bands	Polymorphism %
P1	7	3	42.8
P4	16	14	87.5
P6	9	6	66.6
P7	10	9	90
P8	20	15	75
P9	16	10	62.5
Total	78	57	73.0

Table (5): The genotype-specific RAPD and ISSR markers obtained from the six date palm cultivars.

Genotype	RAPD markers	Total RAPD specific markers	ISSR markers	Total ISSR specific markers
Unknown	A4-548, D10-886, K4-413, P.03-3236, P.03-2261	5		
Frehi	K4-3626, K10-1116	2	P1-1282	1
Oshkingbil	----------	0	P4-2707	1
Azzawy	----------	0		
Siwy	----------			
Karamah	G14-328, K2 -2372, K4-525	3	P1 -2946, P1-4545 , P6-1702 , P4-54.98	4
Total		10		6

The genotype-specific RAPD and ISSR markers for the different date palm cultivars used in the present study were determined (Table 5). Ten out of the forty polymorphic RAPD markers generated were found to be genotype-specific. The highest number of RAPD specific markers was scored for the unknown genotype (5- markers) while the cultivar Karamah and Frehi scored three and two markers, respectively. The genotype-specific ISSR markers for the different date palm cultivars used in the present study are listed in Table (5). The data indicate that 4 ISSR markers distinguished the cultivar Karamah where the Frehi and Oshkingbil cultivars are characterized by one marker each. These markers can be considered as useful markers for the six date palm genotypes and could be used in breeding programs aiming to improve date palm industry.

The RAPD-ISSR based phonogram (Fig. 3) grouped the investigated genotypes into two main clusters. The first cluster included Azzawy and Karamah, the second cluster is divided into two sub-clusters the first one contain the cultivar Siwy and the seconed one is subdivided into two branches the first one have the un-known cultivar and the second one have the cultivars Frehi and Oshkingbil.

Sex identification in date palm

In the present study biochemical (protein and isozyme banding patterns) and molecular markers (RAPD) were applied to distinguish between the male and female trees from the unknown date palm cultivars. The data presented in Fig. (4) show the differences in banding pattern between male and female date palm generated by protein and isozyme analyses. The SDS-PAGE analysis indicates that a clear difference in protein banding was found. Two male specific protein bands with the molecular weight of 72 and 47 kDa and another two female specific bands with the molecular weight of 135 and 55 kDa were detected (Fig 4-A). The Peroxidase analysis indicates a female specific band with the Rf of 0.4, also the band of Rf 0.2 shows intensity difference between male and female date palm as shown in Fig.(4 B). A male specific estrase band with Rf of 0.5 was also detected (Fig 4-C).

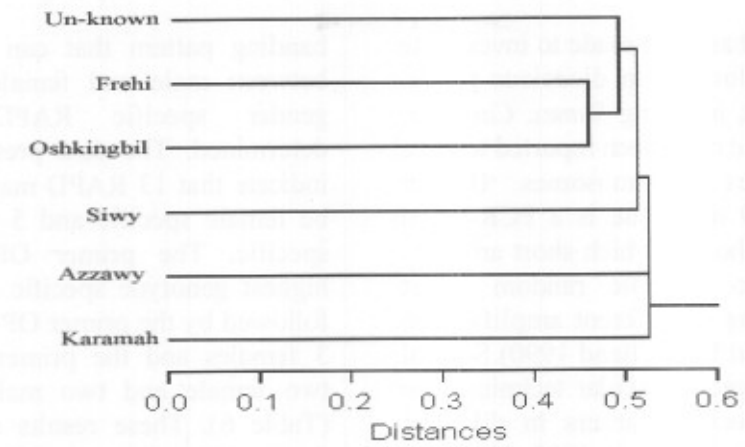

Fig. (3): Clustering of six date palm genotypes based on pooled RAPD and ISSR markers.

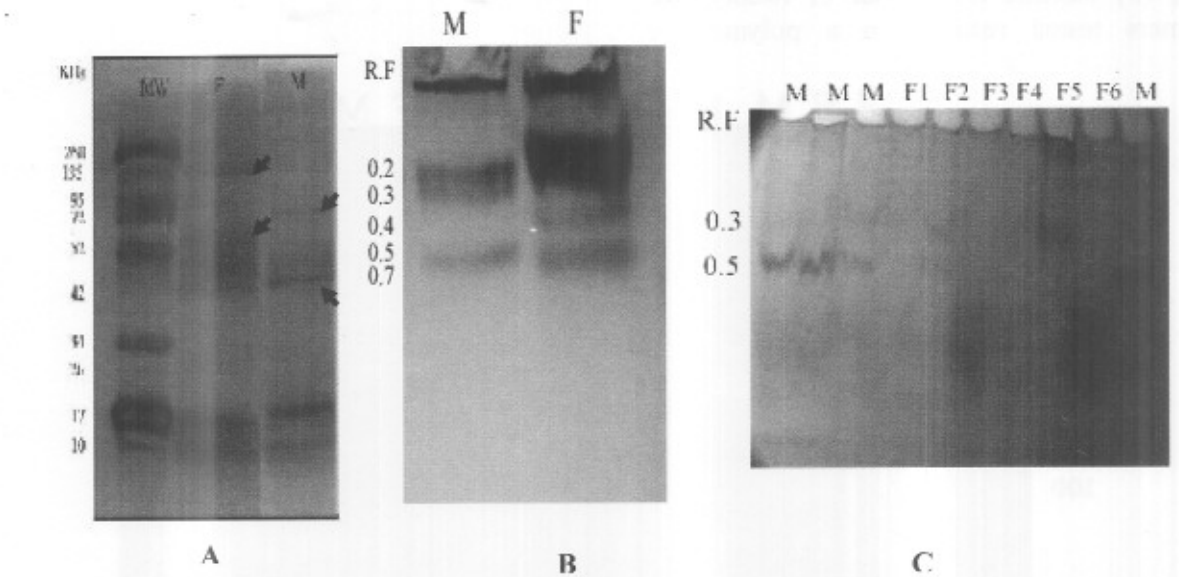

Fig. (4): Biochemical markers for sex identification in date palms. A: Protein banding pattern, B: Peroxidase profile and C: Estrase profile between male and female date palms from the unknown cultivar. MW: Protein marker, F: female and M: male plants.

Much effort has been made to investigate the genetic basis for sex in dioecious plants. Many plant species, including *Rumex, Cannabis, Humulus,* and *Silene* have been reported to have heteromorphic sex chromosomes (Parker 1990). The RAPD technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences, resulting in different amplification patterns (Welsh and McClelland 1990).Several researchers employed molecular techniques to detect the sex specific markers in different plant species (Mulcahy *et al.,* 1992; Hormaza *et al.,* 1994; Biffi *et al.,* 1995; Younis *et al.*, 2008 and Mohasseb *et al.*, 2009).

In the present study the genetic differences between male and female date palm trees were studied at the molecular level using RAPD analysis. The data presented in Fig. (5) indicate that 8 out of twenty two primers tested resulted in a polymorphic banding pattern that can be used to verify between male and female date palms. The gender specific RAPD markers were determined. The data presented in Table (6) indicate that 13 RAPD markers were found to be female specific and 5 markers were male specific. The primer OP-A1 recorded the highest genotype specific marker (4 females) followed by the primer OP-A4 which recorded 3 females and the primer OPD-10 recorded two female and two male specific markers (Table 6). These results are consistent with those of Younis *et al.* (2008) who used RAPD and ISSR analyses to differentiate between male and female date palms and could detect three positive specific markers for females and two for males in RAPD analysis in addition to five positive specific markers for males in ISSR analysis.

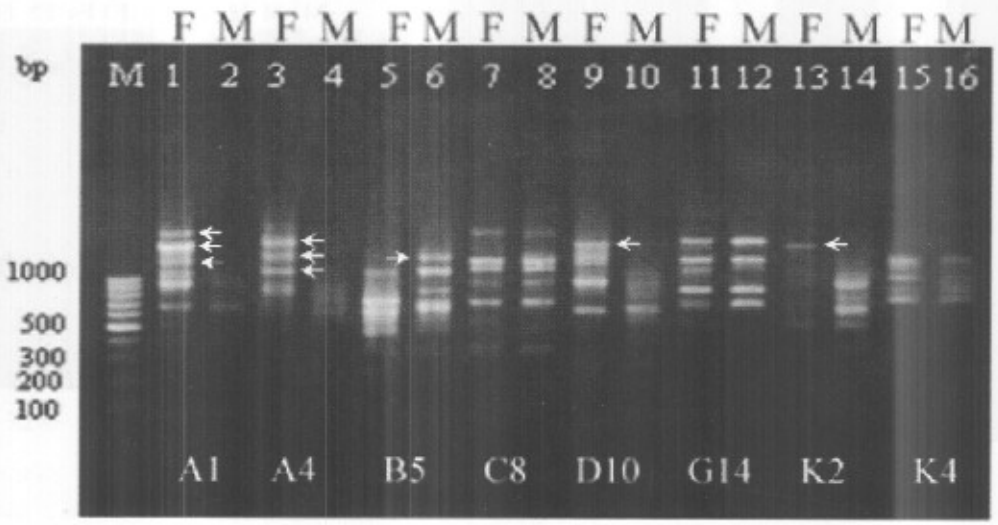

Fig.(5): RAPD banding pattern shows the genetic differences between male and female date palm trees from the unknown cultivar collected from Matroh Governorate.

Table (6): Female and male specific markers as revealed by RAPD analysis.

Primer name	Female specific markers	Male specific markers
OP-A1	3061, 2028, 1684,1412	----------
OP-A4	3567, 2405, 2539	----------
OP-B5	491	2466, 1219
OP-D10	3307, 2539	1734, 857
OP-G14	936	----------
OP-K2	3435	857
OP-K4	1734	----------
Total	13	5

The results of the present study revealed that DNA markers represent an efficient tool for estimating the genetic variability and the genetic relationships among the six date palm genotypes. The markers generated are enough to distinguish between the different genotypes used and can be used to clarify the genetic identity of the unknown genotype. This could represent a useful tool in date palm improving programs. The power of the application of biochemical and molecular markers in sex identification in date palm indicates that both of these markers can be used to differentiate between male and female trees. This could help in facilitating the selection and identification of good male pollinators which can be used in breeding programs aiming to improve the quality of date fruits.

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الملخص العربي

دراسة التباين الوراثي و تحديد الجنس لبعض اصناف نخيل البلح باستخدام واسمات الدنا

رضا علوانى عبد الحليم مغيب*, عبد الهادى عبد الله عبد الهادى* , محمد رضا عبد المجيد احمد**
, احمد جمال الدين محى الدين حسن**
* قسم الوراثة و مركز بحوث و دراسات الهندسة الوراثية- كلية الزراعة- جامعة القاهرة
* *وحدة زراعة الانسجة- مركز بحوث الصحراء – وزارة الزراعة

يعتبر تحديد الاختلافات الوراثية و التعريف السليم لاصناف البلح مهما لكل من المزارعين و كذلك الصناعات القائمة على البلح . يكون التعريف عادة غير ممكن الا اذا انتجت الثمار و نظرا لأن عمر الجيل طويل فان التعريف الوراثى يحتاج للعديد من نتائج الصفات المظهرية للنخيل و التى تتغير بتغير الظروف البيئية. تم فى هذه الدراسة تحديد التباينات الوراثية و كذلك تعريف واحد من اصناف البلح مجهولة الهوية الوراثية والتى تنمو فى محافظة مطروح و كذلك 5 انواع اخرى باستخدام تحليلات كل من الـ RAPD و ISSR . اوضحت النتائج المتحصل عليها ان نسبة التباين الوراثى بين التراكيب الجينية المختبرة كانت 60.2 و73% فى كل من تحليلات كل من الـ RAPD و ISSR على التوالى. يمكن طبقا للنتائج المتحصل عليها التمييز بين مختلف الاصناف المستخدمة فى هذه الدراسة وذلك عن طريق تحديد الواسمات الجزيئية المحدده لكل صنف.حيث وجد ان هناك 5 معلمات جزيئية من معلمات الـ RAPD تميز الصنف المجهول و ثلاثة واسمات (معلمات) تميز الصنف كرامه و اثنان من الواسمات تميزان الصنف فريحى. كما وجد ان 4 واسمات من واسمات الـ ISSR تميز الصنف كرامه وواسم واحد مميز للصنف اوشكنجبيل و واحد ايضا يميز الصنف فريحى. و اوضحت شجرة القرابة الوراثية ان الصنف المجهول شديد القرابة لكل من الصنف فريحى و الصنف اوشكنجبيل .و درست امكانية استخدام كل من الواسمات البيوكيمائية و الجزيئية فى تحديد الجنس فى نخيل البلح حيث اظهرت النتائج ان هناك اثنين من شرائط البروتين ذات الوزن الجزيئى 135 و 55 كيلو دالتون تميزان الاشجار المؤنثة كذلك شرائط البروتين ذات الوزن الجزيئى 72و 47 كيلو دالتون مميزة للاشجار المذكرة. كما اتضح ان واحد من شرائط البيروكسيديز عند الـ RF=0.4 تميز الاشجار المؤنثة عن المذكرة. كما وجد ان شريط الاستيريز المقابل للـ RF=0.5 مميز للاشجار المذكرة عن المؤنثة. كما امكن التمييز بين الذكور و الاناث باستخدام اختبار الـ RAPD و تم تحديد الواسمات الجزيئية المحددة للجنس حيث اوضحت النتائج ان 13 واسم تميز الاشجار الاناث فى حين ان 5 واسمات فقط تميز الاشجار المذكرة عن الاشجار المؤنثة و تعتبر هذة الواسمات مفيدة فى تحديد الجنس فى نخيل البلح فى مراحل نموها الاولى كما يمكن استخدامها فى برامج التربية بهدف تحسين الصفات الثمرية لنخيل البلح.